Light-Induced Cell Death of Retinal Photoreceptors in the Absence of p53

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Purpose. Cell death by apoptosis is essential for normal development and tissue homeostasis, and it is involved also in a variety of pathologic processes. Apoptosis is the final common pathway of photoreceptor cell death in retinal dystrophies and degeneration. So far, little is known about genes regulating apoptosis in the retina. The tumor-suppressor gene product p53 is a potent regulator of apoptosis in numerous systems. However, p53-independent apoptotic pathways also have been described. In this study the authors investigated the role of p53 in the light-induced apoptosis of retinal photoreceptors using mice lacking p53.

Methods. Free-moving $p53^{-/-}$ and $p53^{+/+}$ mice were dark adapted and were exposed to 8,500 or 15,000 lux of diffuse, cool, white fluorescent light for 2 hours. Animals were killed before and immediately after light exposure or at 12 hours in darkness after light exposure. Eyes were enucleated and processed for light and electron microscopy and histochemistry (TdT-dUTP terminal nick-end labeling method). Isolated retinas were subjected to the extraction of total retinal DNA. Electroretinogram (ERG) recordings were performed at all time points.

Results. Morphologic, biochemical, histochemical, and ERG analysis showed that the retinas of untreated $p53^{-/-}$ mice and wild-type control mice were structurally and functionally indistinguishable. After exposure to diffuse white fluorescent light, light-induced photoreceptor cell death was analyzed and was found to be the same in both groups of mice.

Conclusions. These data suggest that light-induced apoptosis of photoreceptors is independent of functional p53. (*Invest Ophthalmol Vis Sci.* 1998;39:846-849)

Cell death by apoptosis is a basic phenomenon in cell biology, contributing to a wide range of physiological and pathologic processes. Morphologically, apoptosis is defined by membrane blebbing, chromatin condensation, and fragmentation of the cell body. On the molecular level, apoptosis is characterized by a decrease of the mitochondrial membrane potential, the activation of proteolytic cascades, and, in most cases, the induction of nucleases that lead to internucleosomal fragmentation of genomic DNA.

In the retina, death by apoptosis is the common fate of photoreceptors in many of the known forms of human and animal retinal dystrophies and degeneration.[1-3] To date, little is known about the molecular regulation and execution of apoptotic cell death in photoreceptors. Recently, it has been shown that the proto-oncoprotein c-Fos may constitutes essential component of light-induced photoreceptor apoptosis.[1]

In this study the role of the tumor-suppressor gene product p53 was investigated for the regulation of the apoptosis of retinal photoreceptors. p53 is a sequence-specific, DNA-binding transcription factor. One of its major functions is to signal DNA damage and other forms of cellular stress and, consequently, to induce either growth arrest at the G1/S border or apoptosis. Whether a cell responds with p53-mediated growth arrest or apoptosis is dependent on the cell type, the differentiation state of the cell, the presence of functional retinoblastoma protein, and the availability of growth factors. There is ample evidence that lack of p53 function may account for the relative resistance to undergo apoptosis after irradiation or treatment with chemotherapeutic drugs, further underscoring the importance of p53 in regulating apoptosis. In the mouse, there is evidence for p53-mediated apoptosis in the lens and retina under conditions of inactivated retinoblastoma protein alleles.[4] To evaluate the function of p53 in the regulation of apoptosis of retinal photoreceptors, we applied our model system of light-induced photoreceptor degeneration[1] to $p53^{-/-}$ and $p53^{+/+}$ animals. We present data that indicate that, in contrast to c-Fos, p53 has no essential role in regulating light-induced apoptosis of photoreceptors.

Materials and Methods

Animals

All procedures concerning animals in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. p53 mutants[5] were bred from heterozygous matings on a C57BL/6 background and were maintained in a 12-hour:12-hour light/dark cycle (lights on at 6 AM) with 10 to 20 lux of light within the cages. Their genotype was determined by polymerase chain reaction analysis of genomic DNA.

Light Exposure

Six- to twelve-week-old male $p53^{-/-}$ mice and wild-type controls were dark adapted for 36 hours, and their pupils were dilated with cyclopentolate 0.5% (Alcon Pharmaceuticals, Cham, Switzerland). Animals were either killed before light exposure or the unanesthetized mice were exposed to 8,500 or 15,000 lux of diffuse, cool, white fluorescent light for 2 hours. During illumination, particular care was taken that the eyes were evenly illuminated.

Tissue Preparation

Mice were anesthetized with CO_2 and were killed by cervical dislocation before and at 12 to 15 hours in darkness after light exposure. Eyes were rapidly enucleated, fixed in 2.5% glutaraldehyde for 12 hours, dehydrated, and processed for light microscopic analysis, or fixed in 2% paraformaldehyde for 2

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hours followed by dehydration and paraffin embedding for TdT-dUTP terminal nick-end labeling (TUNEL) staining (total of $p53^{+/+}$ mice, $n = 6$; total of $p53^{-/-}$ mice, $n = 6$).

TdT-Mediated dUTP Nick-End Labeling Staining

TUNEL was performed with modifications using the in situ cell death detection kit (Boehringer Mannheim, Mannheim, Germany) on 5-μm paraffin sections. DNA strand breaks were labeled with fluorescein and were visualized with a fluorescein isothiocyanate filter (total of $p53^{+/+}$ mice, $n = 6$; total of $p53^{-/-}$ mice, $n = 6$).

DNA Fragmentation Analysis

Retinas were rapidly removed through a slit in the cornea and were frozen in liquid nitrogen. Total retinal DNA was extracted as described.[1] Five to 15 μg of total DNA were analyzed on a 1.8% agarose gel. DNA was visualized at 254 nm by staining with SYBR GREEN (Molecular Probes, Leiden, The Netherlands) and was compared with a 100-bp ladder molecular weight marker (Pharmacia Biotech, Uppsala, Sweden; total of $p53^{+/+}$ mice, $n = 6$; total of $p53^{-/-}$ mice, $n = 6$).

Electroretinogram

Ganzfeld electroretinograms were recorded from dark-adapted $p53^{+/+}$ and $p53^{-/-}$ mice. All animals investigated ($n = 16$) were divided into four groups: $p53^{+/+}$ and $p53^{-/-}$ mice without bright light exposure and $p53^{+/+}$ and $p53^{-/-}$ mice at 12 to 15 hours in darkness after bright light exposure. Anesthesia was induced with a single intraperitoneal injection of xylazine (20 mg/kg) and ketamine (40 mg/kg), and the anesthetized animal was placed on a heating pad (37°C). Silk-AgAgCl-electrodes[6] were placed on the center of the cornea of the dilated left eye, and the reference electrode was placed in the mouth. A platinum ground electrode was inserted subcutaneously into the tail. All manipulations were performed under dim red light using an operating microscope. Light stimuli from a halogen source were presented as pulses of 20 msec over a range of 6 logarithmic units of light intensity (8×10^{-2} to 8×10^{4} cd/m^2). Averaged responses ($n = 4$) were stored digitally on a personal computer and were written on a two-channel chart recorder. A bandpass of 0.03 to 500 Hz was used for a-, b-, and c-waves, but 60 to 500 Hz was used to isolate oscillatory potentials.

Results

Before light exposure, $p53^{-/-}$ and $p53^{+/+}$ mice displayed normal retinal morphology (Figs. 1A, 1B) and normal recordings of a- and b-waves in the electroretinogram (ERG; see Figs. 4A, 4B). Furthermore, the transparency of the ocular media and the light transmission of homogenized crystalline lenses measured by spectrophotometry were found to be similar in $p53^{+/+}$ and $p53^{-/-}$ mice (data not shown). A dose-response relationship of retinal light damage established in $p53^{+/+}$ mice revealed a threshold dose for the induction of apoptosis of 8,500 lux for 2 hours (data not shown). In previous studies we have reported a threshold dose of 5,000 lux for F1 hybrids from C57BL/6 × SV129 mice,[1] which are less pigmented than C57BL/6 mice. After exposure to this light regimen and 12 hours in darkness after light exposure, $p53^{-/-}$ and $p53^{+/+}$ animals showed massive morphologic signs of photoreceptor apoptosis (Figs. 1C, 1D). Light microscopy revealed condensed

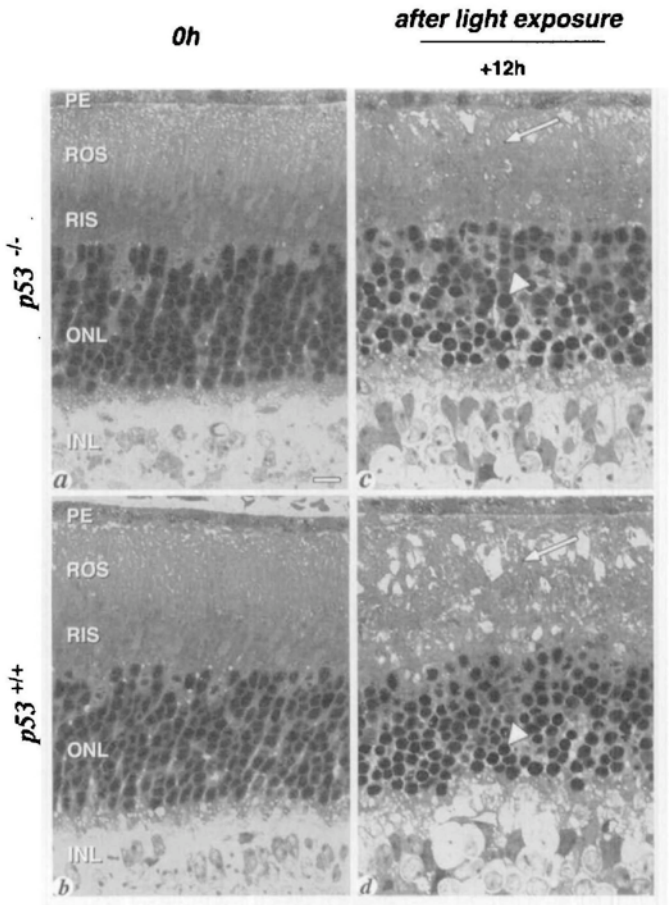

Figure 1. Light microscopy of retinal apoptosis in $p53^{-/-}$ mice (**a**,**c**) and $p53^{+/+}$ mice (**b**,**d**). Retinas were analyzed before illumination (**a**,**b**) or at 12 hours (12h) in darkness after exposure to 8,500 lux of diffuse white light (**c**,**d**). *Arrowhead* shows condensed photoreceptor nuclei in the outer nuclear layer (ONL). *Arrow* shows deteriorated rod outer segment (ROS). PE, pigment epithelium; RIS, rod inner segment; INL, inner nuclear layer. Scale bar, 10μm.

photoreceptor nuclei in the outer nuclear layer, condensed rod inner segments, and deteriorated rod outer segments, which no longer could be differentiated from rod inner segments.

The in situ analysis of DNA strand breaks by the TUNEL method revealed abundant positive nuclei in the outer nuclear layer of both groups of animals at this time point (Figs. 2C, 2D), whereas the retinas of unexposed $p53^{-/-}$ and $p53^{+/+}$ mice were devoid of signals (Figs. 2A, 2B).

At 36 hours after exposure to 8,500 lux for 2 hours, gel electrophoresis of total retinal DNA indicated the occurrence of internucleosomal DNA cleavage (data not shown) in both groups of animals. However, the resultant DNA ladder was at the limit of detection. To increase the extent of photoreceptors simultaneously undergoing apoptosis, and thereby facilitating the detection of internucleosomal fragmentation, we have exposed mice to 15,000 lux for 2 hours. At 12 hours after light exposure, DNA fragmentation was observed in $p53^{-/-}$ and $p53^{+/+}$ mice, whereas the DNA of dark controls was intact (Fig. 3).

The ERG as a light-evoked field potential reflects the functional state of the entire retina, expressed in several components. The a-wave corresponds to the activation of

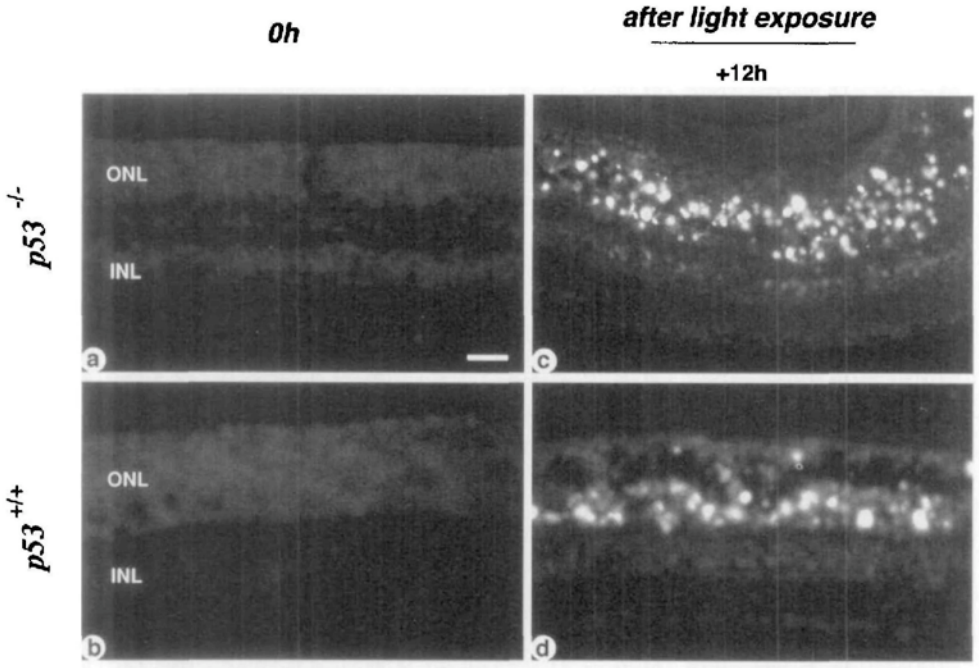

FIGURE 2. Detection of DNA strand breaks in photoreceptor nuclei by in situ TdT-dUTP terminal nick-end labeling (TUNEL). Retinas of $p53^{-/-}$ mice (**a**,**c**) and of $p53^{+/+}$ mice (**b**,**d**) were prepared for TUNEL staining before illumination (**a**,**b**) or at 12 hours (12h) in darkness after exposure to 8,500 Lux of diffuse white light (**c**,**d**). INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar, 100 μm.

photoreceptor cells, the b-wave is generated by second-order neurons in the inner nuclear layer in interaction with Müller glial cells, and the c-wave represents a response of the retinal pigment epithelium to changes in the subretinal space. The ERGs (a-, b-, and c-waves) recorded from $p53^{-/-}$ and $p53^{+/+}$ mice before light exposure were similar (Fig. 4 and data not shown). Recordings at 12 to 15 hours after light exposure to 8,500 lux for 2 hours revealed comparably strong decreases in amplitudes and raised thresholds in $p53^{-/-}$ and $p53^{+/+}$ animals (Figs. 4C, 4D).

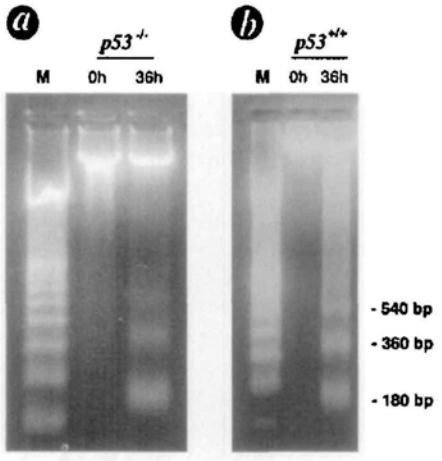

FIGURE 3. DNA fragmentation analysis. Total retinal DNA was prepared from $P53^{+/+}$ and $p53^{-/-}$ mice before (0h) and at 36 hours (36h) in darkness after light exposure. M, 100-bp ladder.

DISCUSSION

To date, little is known about the molecular regulation of cell death in photoreceptor degeneration, and only few genes have been tested for their role in the apoptosis of photoreceptors. In this regard, bcl-2 and bcl-X_L, potent inhibitors of apoptosis in many cell types, were demonstrated to retard photoreceptor apoptosis when overexpressed in the *retinal degeneration* mouse, an animal model for retinal dystrophy.[7,8] Recently, it was shown that mice lacking c-Fos are protected from light-induced photoreceptor cell death.[1] Moreover, numerous cytokines were shown to delay photoreceptor degeneration such as basic fibroblast growth factor in the Royal College of Surgeons rat and a light-damage model using constant illumination.[9]

Here, we report that the tumor suppressor gene product p53 is not essential for light-induced apoptosis to occur. DNA damage is the primary signal triggering p53-dependent apoptosis.[10] However, p53-dependent apoptosis may also occur without the apparent damage of DNA, as in the case of c-Myc-induced apoptosis or under circumstances of inactivated retinoblastoma proteins in the lens and certain parts of the nervous system.[4] Regular retinal morphology of $p53^{+/+}$ and $p53^{-/-}$ mice gives no evidence that developmental apoptotic cell death occurring in the mouse retina is p53 dependent (Fig. 1). This is similar to findings in the mammary gland and neuronal cell lines showing that apoptosis proceeds normally during histogenesis of these tissues in the absence of p53.

Here we show that in $p53^{+/+}$ and $p53^{-/-}$ mice, apoptosis of photoreceptors can be induced by an exogenous stimulus, such as a defined dose of diffuse white fluorescent light, resulting in similar morphologic degeneration and functional

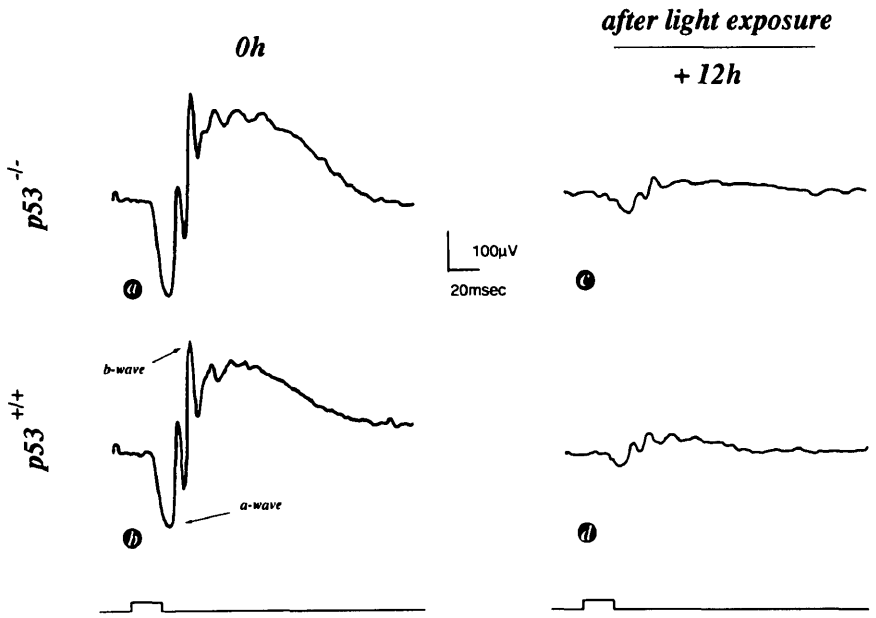

FIGURE 4. Electroretinogram analysis. $p53^{-/-}$ and $p53^{+/+}$ mice were analyzed before (0h) illumination (**a**,**b**) or at 12 hours (12h) in darkness after light exposure (**c**,**d**). The panels show the average of the recordings of four mice. a- and b-waves are indicated. The lowermost trace represents the light stimulus of 20 msec with its upward deflection. Bandpass, 0.3 to 1000 Hz.

impairment of the retina. In both types of animals, photoreceptors die by apoptosis after light exposure, as judged by morphologic criteria, positive TUNEL staining, and internucleosomal DNA fragmentation. Functional damage caused by light exposure is demonstrated by ERG measurement of phototransduction. Whereas ERG recordings from $p53^{+/+}$ and $p53^{-/-}$ control mice showed normal a- and b-waves over a range of 6 log units of stimulus intensity, indicating normal phototransduction, a severe reduction of phototransduction was recorded after light exposure in both types of mice. This implies that the lack of p53 did not alter retinal function. Furthermore, it shows that the extent of photoreceptor damage caused by light exposure does not depend on functional p53, because the morphologic, biochemical, histochemical, and electrophysiological analysis in light-exposed $p53^{+/+}$ and $p53^{-/-}$ mice indistinguishably show the characteristics of photoreceptor apoptosis. Therefore, light-induced photoreceptor apoptosis is p53 independent. Forms of p53-independent apoptosis have been described in a variety of other systems.

For the retina, future studies may reveal whether p53 acts during the apoptotic process of photoreceptors when apoptosis is induced by exposure to ultraviolet light and other DNA-damaging agents such as oxidative stress.

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